A PRELIMINARY INVESTIGATION INTO THE USE OF ENVIRONMENTAL DNA TO DETECT THE PRESENCE OF RARE *EURYCEA* SALAMANDERS IN THE DEVILS RIVER, TEXAS

An Honors Thesis

Presented to the Honors Program of

Angelo State University

In Partial Fulfillment of the

Requirements for Highest University Honors

Bachelor of Science in Biology

by

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May 2014

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ACKNOWLEDGEMENTS

I would like to acknowledge the Angelo State University Department of Biology, the Honors Program at Angelo State University and particularly the ASU Center for Innovation in Teaching & Research (CITR), which provided the funds necessary for me to travel and conduct this work. I would like to acknowledge and thank my faculty advisor, Dr. Michael T. Dixon for taking on the role of thesis supervisor and for his guidance and support on this project. I would especially like to thank Dr. Loren K. Ammerman for serving as a member on my committee and allowing me access to her laboratory and for spending much of her time helping me through this project. I would like to thank Laramie L. Lindsey for the numerous hours she spent in the lab assisting me with this project. Furthermore, I would like to thank and acknowledge the Director of the Honors Program, Dr. Shirley Eoff, for believing in me and presenting me with the opportunity to complete an Honors undergraduate thesis, for participating in my committee, and for being a constant supporter throughout my undergraduate career. Finally, I would like to thank my family and friends who have always been there through all of my endeavors, and I dedicate my accomplishments to them.

ABSTRACT

Preliminary surveys at the Southern Unit of the Devils River State Natural Area (DRSNA-SU) near Del Rio, Texas, suggest that two undescribed species of *Eurycea* salamanders may inhabit this westernmost area of their projected habitat range, but no research has specifically investigated their presence. Members of the genus *Eurycea* found in central Texas are primarily aquatic and inhabit aquifers. Most species of Hill Country *Eurycea* salamanders are suspected to be threatened or endangered. Following an unsuccessful trapping effort, primers were designed from the mitochondrial cytochrome *b* gene that have the potential to be specific to the genus *Eurycea*. As part of an introductory investigation these primers were used to explore the potential of extracting environmental DNA (eDNA) to demonstrate species presence. While these methods did not yield conclusive results, this project contributes to the growing body of work on eDNA as an inexpensive way to monitor freshwater species.

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INTRODUCTION

Historical Overview of the Devils River

For centuries, the Devils River was home to many Native American groups that hunted, fished and gathered in the prosperous river system they knew as Dacate. In 1590 Gaspar Castaño de Sosa, a Spanish explorer leading an expedition out of Coahuila, Mexico, to search for suitable lands to colonize, found himself faced with crossing this large river system and named it Rio de las Lajas, or "River of Rocks" (Texas State Historical Association, 2010; Dearen, 2011). It took two scouting trips to locate a ford and a full day for the group of approximately 170 people to actually make it across. This name stuck for several decades as the Spanish continued to settle the area and have frequent interactions with the local Native American tribes. Although it is not known exactly when the name changed, by 1775 it was known as San Pedro, after the apostle St. Peter. At this point, the river was well known to travelers because it offered water and access from north to south in rugged canyon land (Texas State Historical Association, 2010). Even east-west expeditions followed San Pedro as far as possible before striking out into the desert terrain. In 1848, Texas Ranger Captain Jack Hays struck a course with approximately thirty-six Rangers from San Antonio to El Paso. Along this trek, they found themselves in a constant struggle with the river and as a result named the river the Devils, as was first reported in the Western Texian (Dearen, 2011).

Physical Overview of the Devils River

Today, the Devils River serves as one of the best examples of an ecologically intact

aquatic system in Texas; its clear waters flow over limestone and deposits of sand, gravel and mud (Parent, 2008; Dearen, 2011). It follows along rugged ridges, canyons and grassy banks as it makes its way to Lake Amistad. The United States Board on Geographic Names identifies the source of the Devils in northwest Sutton County at 30°19′40″N, 100°56′31″W, where six moving bodies of water come together: Dry Devils River, Granger Draw, House Draw, Jackson Draw, Flat Rock Draw, and Rough Canyon (U.S. Geological Survey, 1979; Texas State Historical Association, 2010). The river then flows ninety-four miles southwest to its mouth on the northeastern shore of Amistad Reservoir in southern Val Verde County (29°28' N, 101°04' W) (Fig. 1). Thirty-two tributaries feed into the Devils throughout its course, including Dolan Creek, where Dolan Falls is formed, Dark Canyon, Dead Man's Creek, and Satan Canyon (U.S. Geological Survey, 1979; Dearen, 2011; Texas State Historical Society, 2010). A series of springs provides a substantial base of the river's flow. These springs feed out of the Edwards/Trinity Aquifer (Fig. 2).

FIG. 1.−Map illustrating the Devils River from where it leaves Sutton County through Val Verde County and into Lake Amistad. The Devils River Ranch is an older name for the Southern Unit and refers to the land prior to its acquisition by the Texas Parks and Wildlife Department (TPWD). http://www.tpwd.state.tx.us/state-parks/devils-river

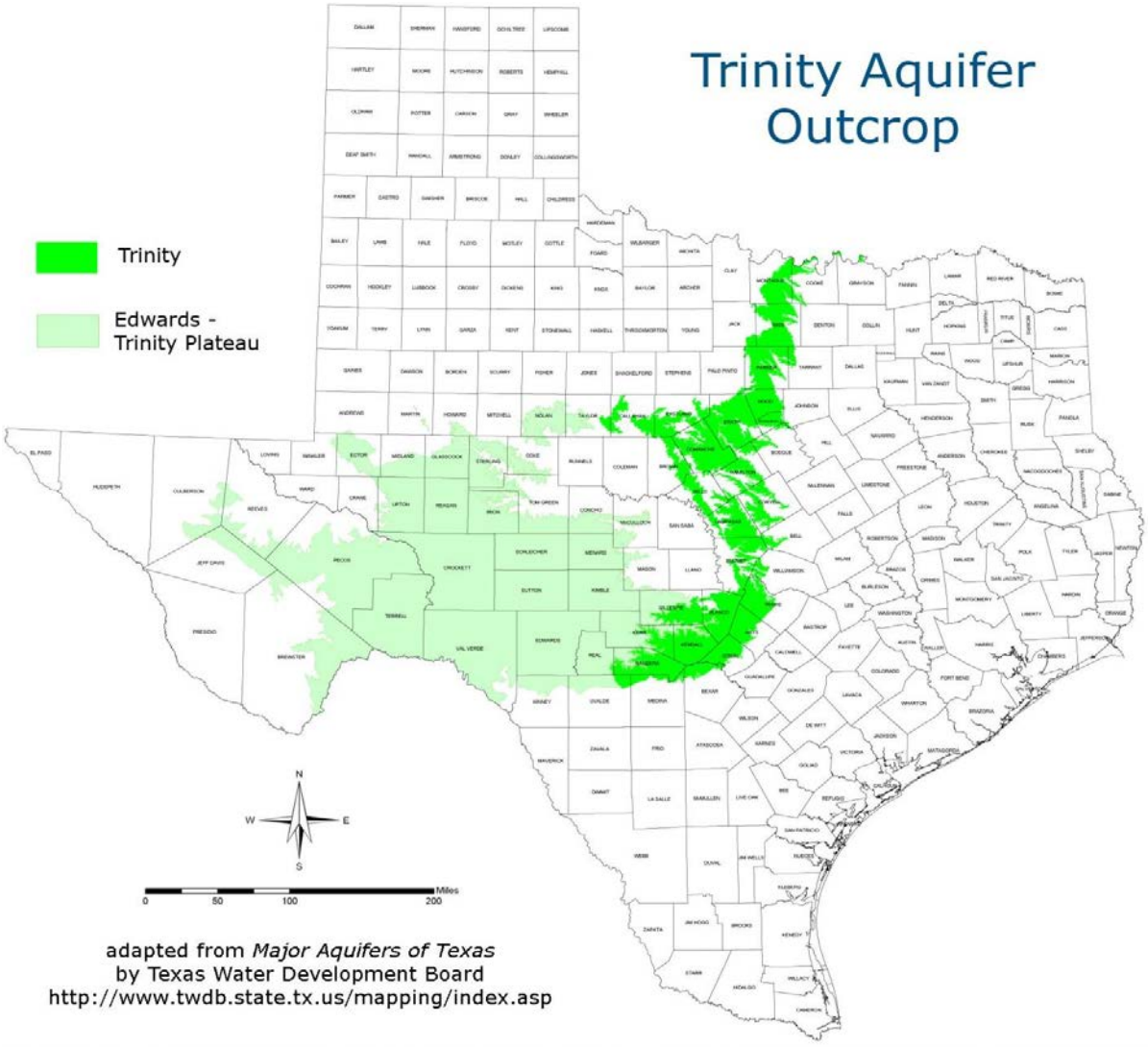

FIG. 2.− Map of the Trinity Aquifer in Texas. The Edwards-Trinity Plateau reaches into Val Verde County, the location of the study site (Eckhardt).

Three types of stream conditions characterize the river: long, deep pools; wide shallow areas; and relatively deep, turbulent rapids. The river remains essentially primitive and unpolluted due to private ownership of the majority of the land around the river. The State Natural Area is situated between multiple biotic zones—the Edwards Plateau, Chihuahuan Desert, and Tamaulipan Thornscrub—allowing for a large variety of habitats and organisms to flourish (Parent, 2008; Dearen, 2011). Vegetation varies from large, dense groves of live oak, pecan, and sycamore trees adjacent to the river, to semi-desert grassland vegetation on the ridges and slopes, and finally numerous springs and seeps teeming with mosses, ferns, herbs and vines. The Devils River State Natural Area is also well known for its historical remains seen in rock art throughout the park (Parent, 2008). The Texas Parks and Wildlife Department (TPWD) currently has one State Natural Area in the northern part of the river by Dolan Falls, and the newly acquired Southern Unit will be approximately 8 miles south (Fig. 3).

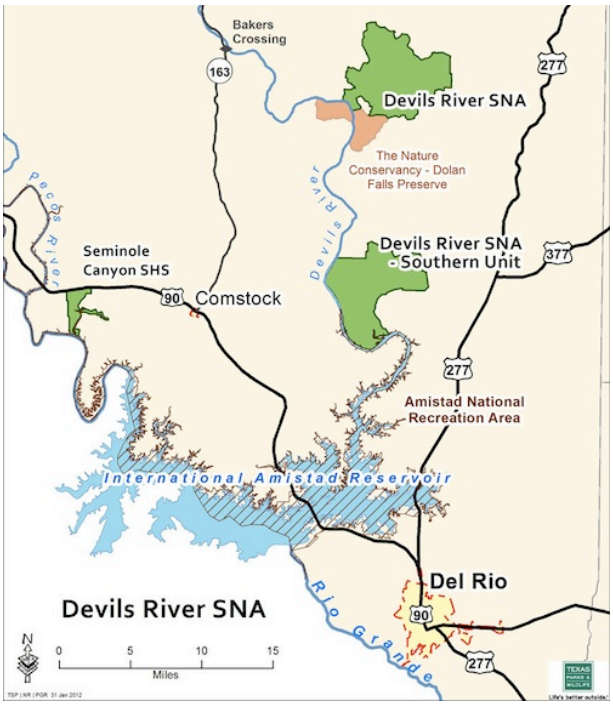

FIG. 3.−Map of the current and future State Natural Areas on the Devils River. http://www.tpwd.state.tx.us/state-parks/devils-river

While the Devils has historically been construed as a desert wasteland, it is home to about 77 species of reptiles and 14 species of amphibians (Dixon, 2000). A 2011 report to the TPWD suggested that two undescribed species of salamanders in the genus *Eurycea* may inhabit this river system (Keith, 2011). Salamanders of the genus *Eurycea*, commonly known as brook salamanders, are distributed throughout eastern and central North America. Where they occur in central Texas, some *Eurycea* inhabit underground aquifers and may never be seen except at the surface of caves or springs. One primary reason this group has gone unstudied is the challenge of finding them. However, studying them is critical as the world faces a rapid global decline of amphibians (Houlahan et al., 2000). Fortunately, new techniques in low density population detection have been developing over the past ten years that can help circumvent some of these issues, such as using environmental DNA (eDNA) to identify species presence in freshwater systems (Goldberg et al., 2011).

Amphibian Conservation

Over seven-thousand species of amphibians inhabit the world today. Tragically, one-third of these species are currently threatened or endangered (Houlahan et al., 2000; IUCN, 2013). The United States, home to 297 amphibian species, is not exempt from these alarming declines in populations. Since the turn of the millennium, numerous research studies have focused on the cause of these global declines. One such study by Young et al. (2001) highlights the main issues faced by amphbians worldwide. They cited habitat destruction, which encompasses habitat modification and habitat fragmentation, as the primary cause for declines. Habitat modification largely refers to the clearing of lands for settlement or cultivation. Habitat fragmentation can refer to any barriers that can divide a habitat such as roads or changes in enviornment that prevent normal dispersal of the population (Young et

al., 2001). They also explored factors that may not be completely understood yet, such as climate change, introduced species, UV-B radiation, chemical contaminants, soil toxins, trade, and synergisms (Kiesecker et al., 2001; Young et al., 2001). Each factor can cause harm to a population through a different process, and any combination of these situations would constitute a synergism. Climate change could result in disruptions in microclimate or macroclimate conditions. UV-B radiation can cause damage to amphibian cells just as in humans, causing increased mutation rates, lesions, egg mortality, and susceptibility to disease (Kiesecker et al., 2001). The chemical and soil contaminants generally encompass the same concerns and can contribute to direct mortality of eggs and adults or create barriers to dispersal. Trade as a factor only applies to those organisms that are collected for the pet trade or culinary, medical, and biological supply markets; however, this affects quite a few species and can make direct impacts on a population. One final factor considered by Young et al. (2001) that is especially applicable today is disease. New diseases such as chytridmycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) and viruses of the family Iridoviridae are decimating amphibian populations globally (Young et al., 2001; Whittaker et al., 2013). There have been particularly devastating *Bd*-related declines in South and Central America, Spain, Australia, and California (Whittaker et al., 2013). There is debate as to whether this is a new disease or simply one that has increased in virulence as amphibian populations become more vulnerable because of habitat destruction and fragmentation. Regardless of this, chytridmycosis is alarming to the scientific community because it can cause rapid decline in species that have otherwise been stable (Berger et al., 1999; Whittaker et al., 2013).The viruses of the family Iridoviridae (ranaviruses) have not received as much press as *Bd* but are still a cause of mass mortality in frogs and salamanders. Both *Bd* and

ranaviruses are partially if not mostly mediated by human interference and invasion of a habitat with the fungus or virus on them (Whittaker et al., 2013). There is no known cure to *Bd* that would be practical in the field, so currently researchers are focusing on targeted detection methods so the infection can be caught early and humans can intervene to save a majority of a population (Berger et al., 1999). A final consideration for conserving amphibian biodiversity is their role as bioindicators. Guzy et al. (2012) found that presence of five different frog species in a wetland ecosystem served as reliable indicators of wetland integrity. Another study indicated the same may be true in the case of aquatic salamanders (BIO-WEST, 2005). Salamander abundance was measured over several years and trends were found between abundance and other environmental factors such as flow rate and silt deposition. These studies establish the critical importance of amphibian population monitoring.

Family Plethodontidae

The family Plethodontidae, or the lungless salamanders, is a unique group that contains some of the smallest members of the salamander family that can be found worldwide (Vieites et al., 2011). As their common name suggests, they have no internal lung structure and breathe through their skin. Researchers have proposed that the loss of lungs is an adaptation for their life in the water; in larval stages, having an air sac like a lung could cause the salamander to float, displacing it from its primary food source and threatening its survival (Fong et al., 1995). Adults reach a maximum size between 2.5-30.5 cm depending on the species, and the larval stages are generally much smaller than the mature form (Welsh and Droege, 2001). With a large portion of these salamanders living in moving water, oxygen is constantly turned over and there is little selection for keeping lungs (Fong et al., 1995).

Another physical characteristic of the family Plethodontidae is having four toes on the front limbs and four or five toes on the hind limbs with a medium length tail. The head is modified for wedging under rocks and burrowing in silt on the streambeds. Plethodontids also have a modified long tongue that can be flicked quickly to capture prey (Welsh and Droege, 2001).

As for their diet, most of the lungless salamanders eat small crustaceans, insects, and sometimes worms. Larger species have been known to eat smaller salamanders as well (Holomuzki, 1980). On the whole, little is known about Plethodontid reproduction and their daily behaviors. It is known that they like to live in groups and some species are highly territorial. For the terrestrial species, they are hidden during the day and active at night, with peak movement at sunset (Holomuzki, 1980). They rely on covertness as opposed to speed to avoid capture. Contrastingly, water-dwelling species have more speed than their land-dwelling cousins. Depending on where they live during their adult life and if they have a larval stage or not, females will lay their eggs in shallow water along streams or on land, hidden under rocks or in moss layers. Several species in the family also demonstrate parental care and females will remain to protect the clutch and will not eat during this maternal stage (Jaeger and Forester, 1993).

Genus *Eurycea*

Within the family Plethodontidae, there exists the genus of *Eurycea*. The online reference Amphibian Species of the World 6.0 currently recognizes 28 species of *Eurycea* (Frost, 2014). Of these 28 species, 15 species live their entire lives underground, have little to no skin pigmentation, and may or may not have vestigial eye structures (Fong et al., 1995; Frost, 2014). These cave salamanders are all neotenic, or remain in a larval form for their entire lives (Baker, 1961). The range of *Eurycea* in Texas is confined to about fourteen

counties, all a part of the Edwards Plateau (Fig. 4). The easternmost county where specimens of *Eurycea* have been collected is Williamson County, and the westernmost is Val Verde County.

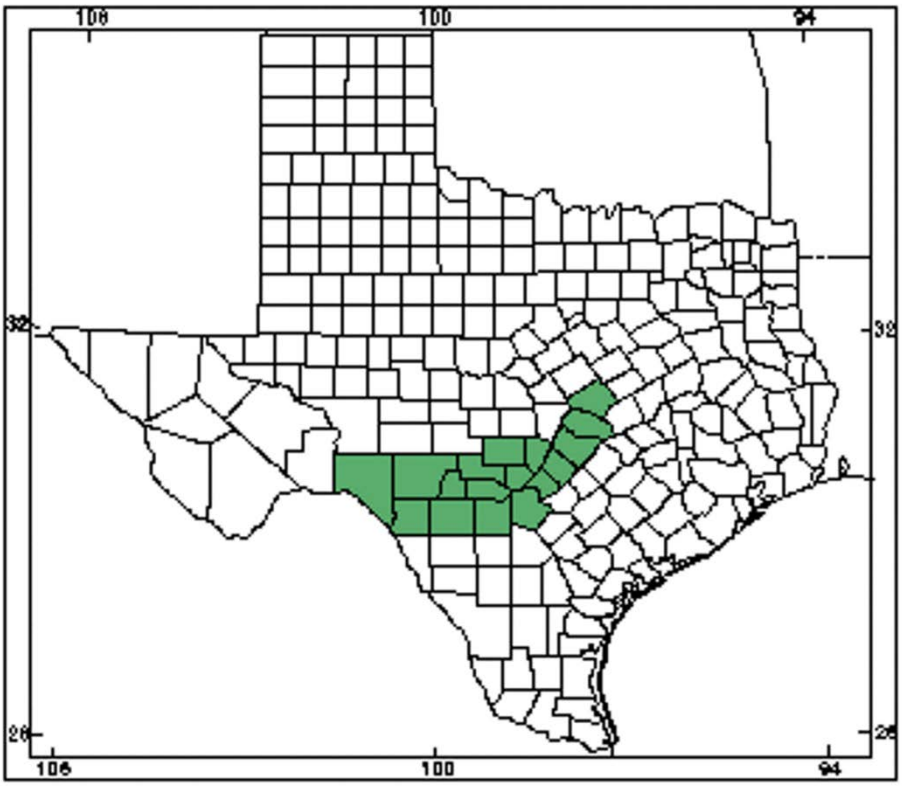

FIG. 4.−Distribution map of 28 species of subterranean *Eurycea* salamanders in Texas (Salamanders of Texas)

Baker (1961) published an “updated” key to the neotenic *Eurycea* in the Edwards Plateau region and lamented the fact that the morphological differences between species are so similar it is nearly impossible to key them out. At the time when the article was published, scientists were only just beginning to grasp that these Plethodontids were separated in different caves long enough for the species to diverge through the process of allopatric speciation (Baker, 1961).

In more recent times, the phylogenetic tree of *Eurycea* has been scrutinized using allozyme loci and mitochondrial DNA studies (Chippindale et al., 2000). The work was done to characterize genetic variation and diversity, isolate species boundaries, and assemble the phylogenetic history of Plethodontidae in central Texas. From this study, researchers discovered that the genus *Typhlomolge*, a group previously separate from *Eurycea*, was actually phylogenetically nested within the central Texas *Eurycea*. Furthermore, they concluded that species in the northern counties of the Edwards Plateau such as Bell, Williamson, Travis, Blanco and Hays County diverged earlier than the southeast and southwest regions (*E. troglodytes* complex). The complex found in the southwestern counties (e.g. Val Verde, Edwards, Kinney, Uvalde and Real) are suspected to be most closely related with the southeast group, which is comprised of the remaining counties of Kerr, Bandera, Medina, Gillespie, Kendall, Comal and Bexar (Fig. 5). By examining the caves, researchers found that while there was some potential for gene flow to occur, all the populations were essentially isolated (Chippindale et al., 2000). The results of the allozyme studies on specific populations revealed that intrapopulation homozygosity levels were significantly high, possibly due to the founder effect, which is characterized by a loss of diversity when a portion of a larger population establishes a new population in a new area.

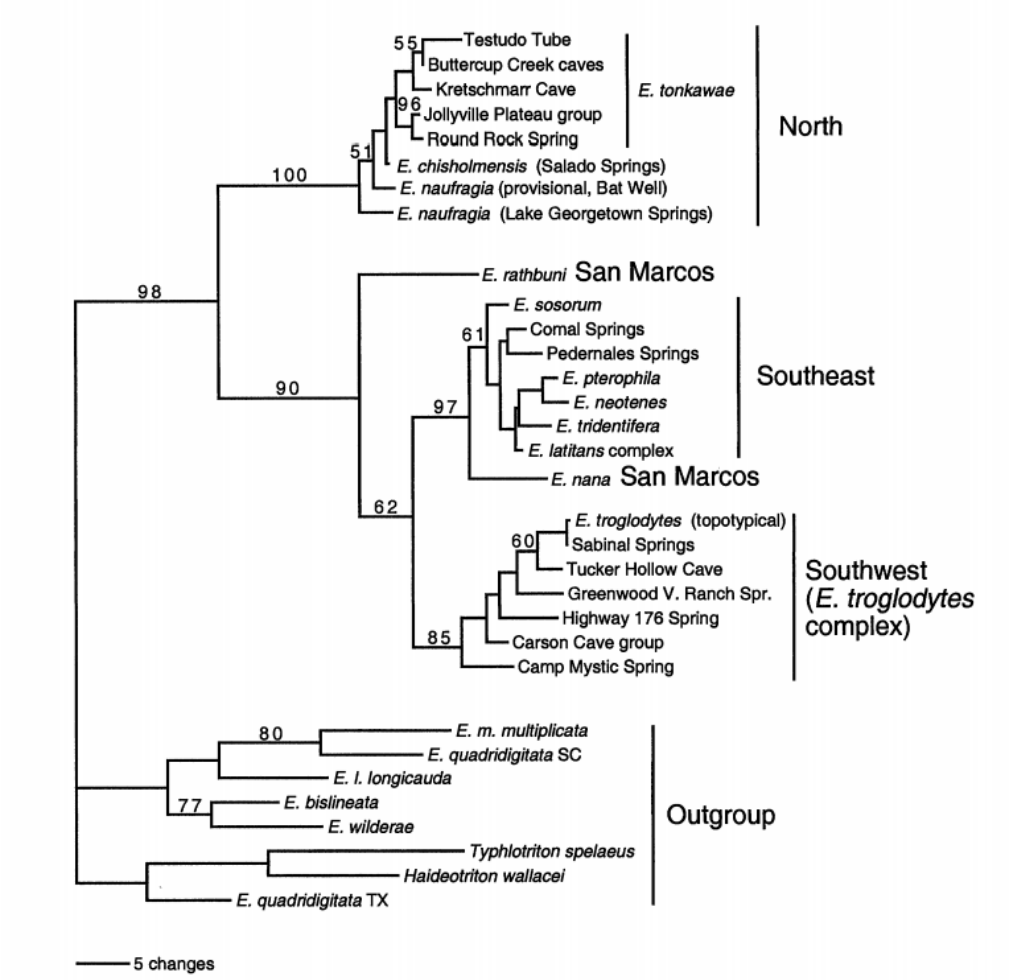

FIG. 5.−Phylogenetic tree of central Texas plethodontid salamanders based on combined cytochrome *b* and allozyme sequence data (Chippindale et al., 2000). *Eurycea* sp. 7 and *Eurycea* sp. 10 are expected to be within the *E. troglodytes* complex.

Another project was undertaken recently to reevaluate the phylogenetic tree of all plethodontids. The results of the work done by Vieites et al. (2011) found a close relationship between the subclade containing *Eurycea* and a subclade comprised of tropical plethodontids, demonstrating that New World Plethodontids are more related to each other than to Old World groups (Vieites et al., 2011). While Vieites et al. (2011) studied plethodontids from around the world, the information they gathered regarding North American *Eurycea*

validated prior studies done by Chippindale et al. (2000), placing *Eurycea* in one of the more recently diverged groups.

Because lungless salamanders breathe through their skin, they suffer dramatic effects from water pollution. Without a protective skin layer, nearly any trace amounts of toxins will be absorbed by the salamanders. The International Union for Conservation of Nature (IUCN) lists one species of lungless salamander as Extinct, 31 as Critically Endangered, 83 as Endangered, 54 as Vulnerable, and 36 as Near Threatened (IUCN, 2013). As habitat fragmentation/destruction, climate change, UV-B radiation and disease threaten amphibians as a group, salamanders may be lost in the mix (Kiesecker et al., 2001).

With concern for their survival rising, the study of salamanders has increased across the board. Being at the edge of the Edwards Plateau, which is one of the most species rich areas for cave-dwelling salamanders in the world, the Devils holds great promise to be home to aquatic salamanders. Val Verde County is currently listed as the westernmost and southernmost end of the range of these salamanders; however, there is some evidence to support their presence. First, the report by Keith (2011) to the TPWD suggested two species of *Eurycea* may inhabit the river. Then even more compelling evidence can be found in the work published by Chippindale et al. (2000), where they state that *Eurycea* specimens were collected by a man named Riley Nelson and his team north of Del Rio in springs in the Devils River drainage. Unfortunately, this discovery followed previous unsuccessful attempts to collect specimens and was too late to be included in the molecular study (Chippindale et al., 2000). There was no citation in the paper specifically referring to this capture and no mention if Riley Nelson published any work regarding the supposed *Eurycea* specimen; it was only mentioned that the specimens seemed consistent with other surface populations

from the southwest. The Devils River has one of the greatest base flows of any Texas river, owing to the many springs that bubble up from underneath the river (Brune, 2002). The TPWD Element Occurrence Report for August 30, 2011, and the TPWD Wildlife Diversity Tracked Animals published that the Dolan Falls salamander, which was referred to as *Eurycea* sp. 10, and the Edwards Plateau spring salamander, which was referred to as *Eurycea* sp.7, may be present or have been found in the Devils River State Natural Area South Unit (Keith, 2011). This area will soon be turned into a public use area, so knowledge of the presence of these species is critical for wildlife management planning. Both species are scientifically undescribed, and if specimens were collected, all morphological data should be analyzed and tissues taken in an effort to describe these species. Furthermore, studies should be conducted to gain better understanding of the life cycle and behavior of cave salamanders in the genus *Eurycea*.

Extracting DNA from Water

Several studies from the last five years have implemented a new technique for locating rare species that are in low density in freshwater environments. These papers have extracted environmental DNA (eDNA) from water samples and successfully amplified the fragments. Goldberg et al. (2011) emphasized that research into the ecology and range of several stream species can be extremely challenging due to a multitude of reasons such as complexity of topography and vegetation, water flow rates, low density populations, and use of microhabitats, which are areas that are limited in size and have a character that separate them from the larger surrounding terrain. Cave-dwelling *Eurycea* use microhabitats found at the mouth of caves or springs and exist on the surface at low densities. Goldberg et al. (2011) go on to suggest that detection of species using eDNA could increase accuracy by comparing

sequenced samples to a database and decrease field collection costs because researchers are simply collecting water samples. Thomsen et al. (2012) suggest that eDNA may be the method by which researchers can get to large-scale comparative validation of species distribution and abundance in a quick, cost-effective and standardized way. However, Goldberg et al. (2011) caution that sampling design should be guided by the ecology of the target organism to maximize chances of detection. In the case of this research, for example, water samples were collected near the mouths of springs to increase detection chances. Pilliod et al. (2014) explored several environmental factors that could influence eDNA detection: different light and temperature conditions; the rate and amount of eDNA produced by individuals; the effects of animal density and the amount of time the population has spent in the area of detection. Their results supported what most other papers have suggested, that detection of species is possible even after a species has only been present for a few hours and that eDNA degrades beyond the point of detection within one to two weeks and that rate is correlated to the condition of the environment. Ultimately, all the literature examined suggests that eDNA methods have great promise to become a standardized, sensitive tool that is widely used to document abundance and distribution of aquatic species over time and location (Goldberg et al., 2011; Thomsen et al., 2012; Pilliod et al., 2014).

Purpose

After discovering that low density populations could be detected in fresh water ecosystems by extracting eDNA from water, it was hypothesized that by designing primers specific to the genus *Eurycea* and using eDNA procedures conducted by Ficetola et al. (2008) presence or absence of the threatened salamander species *Eurycea* sp.7 and *Eurycea*

sp. 10 could be determined in the Devils River through detection and sequencing of eDNA of the target species.

MATERIALS AND METHODS

Field Methods

In the summer of 2013, three trips were taken to DRSNA-SU to capture live specimens. Prior to entering the field, an effort was made to find a source that mapped all the springs throughout the river, but there was no such document readily available. The general strategy was to observe the river and look for potential spring sites, followed by swimming to feel for colder water and look for potential spring openings with swimming goggles. Once a potential site was identified, usually defined as having a relatively slower flow rate, some sort of rocks or foliage for coverage, and a different soil type than the surrounding area, standard minnow traps were set as suggested by Heyer et al. (1994). The traps were baited with either pistachios, earthworms, or amphibian food purchased from Walmart.

On the first trip, five traps were set for three nights, or fifteen trap nights. On the second trip, only three traps were set for two nights, or six trap nights. The same methods were used but in different locations, all which were found after setting out from the Summer House (29°41'02.0"N, 101°00'05.9"W). On the final trip, an approximately 35 ft. deep hole that was about 7 ft. wide (29°40'46.4"N, 101°00'07.7"W) was identified as a potential site because of the change in soil at the bottom and the fact that there appeared to be a side entrance to the bottom of the hole. Four traps were set at various depths along one string baited with only the amphibian food for four nights or sixteen trap nights. It was hypothesized that finding positive DNA results in areas of DRSNA would help future capture attempts and trapping efforts ceased.

Primer Design

Multiple sources found the cytochrome-*b* mitochondrial gene (cyt-*b*) useful to identify salamander species using PCR methods (Chippindale et al., 2000; Hillis et al., 2001; Ficetola et al., 2008; Thomsen et al., 2012). Partial sequences of the beginning of the cyt-*b* gene of about 12 *Eurycea* salamander samples were downloaded from GenBank, all starting from base pair one and ending somewhere near 1,000 base pairs. These sequences were aligned in the software Molecular Evolutionary Genetics Analysis (MEGA) 5.2.1 (Tamura et al., 2011) citation). New primers were created by selecting various sequences between 23-27 bp in length at various distances from the forward primer in order to create fragments that were less than 100 bp long (Goldberg et al., 2011). The sequences were then compared to NCBI's Nucleotide BLAST to see if the sequence was unique to *Eurycea*. NCBI's Primer BLAST was utilized by inputting the cyt-*b* gene of a sample of *Eurycea neotenes* (GenBank accession: AY528400) and selecting for the results to be specific to *Eurycea*. After this first round of elimination, the primer pairs were analyzed in OligoAnalyzer 3.1 (Integrated DNA Technologies, USA). This program shows important information for primers such as GC content, melting temp, hairpins, and self-dimers. After checking all these variables, 5 primers were designed and ordered: Forward 3, Forward 7, Forward 10, Reverse 7, and Reverse 10 (Table 1).

TABLE 1.− Primers designed from the mitochondrial cyt-*b* gene for the genus *Eurycea*

Primer Name	DNA Sequence (5'-3')	Location on gene (bp)
Euryc CytB Forward 3	CATACCTCAAAGCACCGAAGCAT	905-927
Euryc CytB Forward 7	AAAGCACCGAAGCATGTCATTTC	913-935
Euryc CytB Forward 10	ACCTCAAAGCACCGAAGCATGTC	908-930
Euryc CytB Reverse 7	TACCTGGCTGACCCCCAATTCAA	1007-991
Euryc CytB Reverse 10	CTTGGCTGACCCCCAATTCAAGT	1005-989

Water Collection and eDNA Extraction

Following the collection procedures set by Ficetola et al. (2008), water was collected from Barton Springs Pool in Austin, Texas, which is home to the Barton Springs Salamander (*Eurycea sosorum*) as a positive control. Water was taken from the Barton Springs diving board (DB1 and DB2) and the deep end of the Barton Springs pool (DEEP1 and DEEP2). For a negative control, water was collected from two tanks in the Biology Department at Angelo State University. The first was Dr. Michael Dixon's office tank, which contained a Barred Tiger Salamander (*Ambystoma mavortium*) and guppies (*Poecilia reticulata*) [DIXON1 and DIXON2]. The second was a tank in the Angelo State zoology laboratory, which contained assorted native fish (*Lepomis sps)* [ZOO1 and ZOO2]. The Barred Tiger Salamander and fish served as negative controls because they are outside the genus *Eurycea*. Water was collected in 15 ml samples from each location and labeled (Table 3). The water from the Devils River was collected in two locations, one towards the north of the Southern Unit and one towards the south of the Southern Unit. The northern sample (DRSNA-N) was collected by the "Take-Out Point" (29°43'01.1" N, 101°01'18.4" W) and the southern sample

(DRSNA-S) was collected by the Summer House (29°41'02.0"N, 101°00'05.9"W). Immediately after collection each sample was treated with 1.5 ml sodium acetate 3 M and 33 ml absolute ethanol (Ficetola et al., 2008). Treated samples were then frozen at -20° C until DNA extraction. For extraction, samples were centrifuged at 6° C at 5500 g for 35 min. Following this step, the supernatant was discarded and the pellet underwent a classical DNA extraction using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA).

The products from the extraction were separated on a 0.8% agarose gel by electrophoresis and analyzed, and this step was performed to verify the methods and to compare concentrations of DNA collected from the tank samples to the Barton Springs samples. Following electrophoresis, the samples were analyzed using a Qubit® fluorometer **(**Life Technologies, CA) with a High Sensitivity (HS) assay to quantify the concentration of DNA extracted.

PCR

Two primers were selected at random and six samples (DIXON1, DIXON2, ZOO1, ZOO2, DEEP1, DEEP2, DB1, DB2) underwent PCR with an annealing temperature of 55° C. The profile for PCR consisted of a denaturation step at 95° C for 10 min, and then the samples were cycled for 55 times at 95° C for 30 sec, 55° C for 30 sec, and 72° C for 2 min. The final step was at 72° C for 10 min (Ficetola et al., 2008). Products were separated on a 1% agarose gel. PCR was performed again using an annealing temperature of 48° C, as opposed to 55° C. Products were separated on a 1% agarose gel again. After a switch to a lower range ladder (GeneRuler Ultra Low Range DNA Ladder, Thermo Scientific, MA) a 3% agarose gel was used on all subsequent PCR reactions. The next PCR reaction included the four Barton Springs samples (DEEP1, DEEP2, DB1, and DB2) and the two Devils River

samples (DRSNA-N and DRSNA-S). The negative controls were excluded to conserve resources. Products were separated on a 3% agarose gel. From the six primer combinations used, the best two pairs were selected (Forward 3 and Reverse 7; Forward 7 and Reverse 7) and used for another PCR reaction that included the negative controls along with all other previously used samples, and this reaction was followed by gel electrophoresis and quantified with an HS assay in Qubit® (Life Technologies, CA). The four most prominent bands that had sufficient product from this gel were sequenced twice with the Forward 7 and Reverse 7 primers using the DTCS Quick Start Master Mix (Beckman-Coulter) in a Beckman-Coulter CEQ8000 Genetic Analysis System DNA sequencer (Pasadena, CA). Sequencing was carried out in accordance with manufacturer's specifications for a half volume reaction.

RESULTS

Trapping

Collection of the minnow traps during the first and second trips revealed that only fish were captured. During the final trip, nothing was caught in any of the traps.

Primer Design

New primers were designed in an attempt to make primer sets that were specific to the mitochondrial cyt-*b* gene in the genus *Eurycea*. Partial initial sequences of 12 species of *Eurycea* were studied and submitted to NCBI's Prime BLAST, generating 10 primer pairs. Primers that were between 23-27 bp, had minimal chance of self-dimerization, heterodimerization, hairpinning, and relatively low GG content after being analyzed through Integrated DNA Technologies' OligoAnalyzer were considered (Table 1).

Only those primer pairs that were less than 100 bp apart were ultimately selected (Table 2). There was concern that eDNA from the water would most likely be short fragments due to all the agents in the environment that can damage DNA so a short primer pair was preferable (Ficetola et al. 2008). The results of this effort yielded five primers: Forward 3, Forward 7, Forward 10, Reverse 7, and Reverse 10 (Table 1, Fig. 6).

Extraction of eDNA from Water

Very low yields of environmental DNA were obtained from all water collected (Table 3). The most DNA was obtained from sample DEEP2 (0.0656 ng/µl).

PCR

The primer pairs that produced the most prominent bands were Forward 3 and Reverse 7, and Forward 7 and Reverse 7. All samples produced products very close to 100 bp in length except for DEEP1 and DEEP2, although the bands were somewhat diffuse.

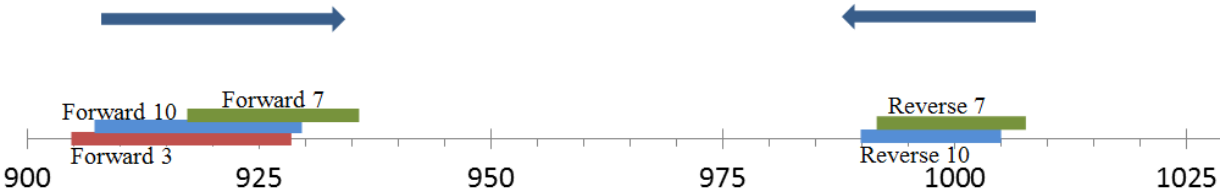

FIG. 6.−Illustration of the location of the designed primers where numbers correspond to base pairs in the cytochrome *b* gene in *Eurycea.*

TABLE 2.−Expected length of PCR products with each possible primer combination.

Possible Primer Combination	Length of Product (# of base pairs)
Forward 3-Reverse 7	64
Forward 7-Reverse 7	56
Forward 10-Reverse 7	61
Forward 3-Reverse 10	62
Forward 7-Reverse 10	54
Forward 10-Reverse 10	59

TABLE 3.−Comparison of water samples' sources and the amount of DNA detected in each.

Water Source	Expected Traces of DNA	Sample Name	Total DNA after Extraction (ng/µl)	Concentration of PCR Product (ng/µl)
Barton Springs	Barton Springs Salamander (*Eurycea sosorum)* Human Assorted Fish	DB1	0.017	1.53
		DB2	0.015	5.62
		DEEP1	0.019	-
		DEEP2	0.0656	-
Devils River	*Eurycea* sp. 7 & sp. 10 Assorted Fish Assorted Mammals	DRSNA-N	<0.50	2.11
		DRSNA-S	<0.50	1.25
Zoology Lab Fish Tank	Native Texas Fish (*Lepomis* spp.)	ZOO1	0.0296	2.89
		ZOO2	0.0201	0.567
Dr. Dixon's Office Tank	Barred Tiger Salamander (*Ambystoma mavortium*) Guppies (*Poecilia reticulata*)	DIXON1	<0.050	0.44
		DIXON2	0.016	1.33

Sequencing Genes

Although the PCR yield was low, the sequencing protocol was attempted for four PCR samples that had the most DNA in them (DB2, ZOO1, DRSNA-N and DRSNA-S). The results of this sequencing step did not produce good quality sequence despite repeated attempts.

DISCUSSION

The results of this study were inconclusive. From the first PCR reaction, trace amounts of DNA was detected in all samples, which could imply that the primers were never specific to *Eurycea* as they should have been; however, the values were so low that they probably are not significantly different from zero. Unfortunately, with no successful sequences isolated and by not working with a known specimen, many unknown variables could not be accounted for. The next step in this work would be to systematically alter and change the conditions of extraction and PCR to optimize DNA concentrations. With such a small amount of DNA extracted and amplified through PCR, there probably was never enough for successful sequencing. It is possible, though unlikely, the conditions of the sequencer were not correct or that the solutions were contaminated in some way, but if there was not a sufficient amount of DNA to sequence this would not have impacted the results. Potentially, the samples could be sent away to be sequenced in another lab, but the small amount of DNA will probably not be enough. Without sequencing, it is extremely difficult to demonstrate species presence.

An additional concern regarding this study may tie back to the water collection itself. Goldberg et al. (2011) and Pilliod et al. (2014) both mention in their respective works that collection of eDNA can be greatly impacted by seasonal changes in the target species. If they have a reduced metabolism or are less active in the winter, this may skew detection rates. Getting as close to the habitat as possible may decrease some of these concerns.

Future researchers may want to consider optimizing the PCR protocol to generate higher amounts of PCR product and should also include a known *Eurycea* specimen for a positive control. Performing this research without a known positive control did not allow certainty that the primers were selectively amplifying the cytochrome *b* gene from *Eurycea*.

The only certain result of this study is that the storage methods and extraction methods proposed by Fiectola et al. (2008) were effective in protecting the DNA from complete degradation for months and allowed extraction for this study. It was also certain that the primers designed for this study were binding to DNA as shown through successful PCR trials, though the source of the DNA remains unclear.

CONCLUSION

As stated previously, there unfortunately was no conclusive evidence from this study, though it does contribute to the growing body of work on eDNA and highlights some of the potential pitfalls that researchers can encounter. However, the lack of detection of this species does not necessarily demonstrate its absence. The literature of Keith (2011) and Chippindale et al. (2000) certainly support presence at some point in time, though it is possible the species have migrated or are extinct in that specific location. Knowledge of these species' presence or absence would contribute to the general body of amphibian conservation knowledge. This study should be considered a preliminary test of the use of eDNA to assess species presence. Once the issues surrounding the primers are resolved, this work would also add to the body of eDNA research being done and if the primers are shown to work for all *Eurycea*, would be useful for any work done on *Eurycea* across the state. If a known specimen of an aquatic *Eurycea* salamander could be obtained to test the primers on, this would offer insights into the limitation of this particular study.

Such work would be a very important source to future studies on the ecology and environmental changes of the Devils River as it becomes more readily accessible to the public. Thomsen et al. (2012) present the case that with the rapid decline of biodiversity and amphibians in particular, there is a call for fast, reliable and effective methods to monitor threatened species, which eDNA has the potential to become. Another consideration for the importance of continuing this type of work is amphibian presence has been found to be an indicator of an environment's overall health (BIO-WEST, 2005; Guzy et al., 2012). Due to their sensitivity to ecological changes, determining the presence or absence of these *Eurycea* species will provide a baseline for future comparisons. If *Eurycea* sp. 7 and *Eurycea* sp. 10

are present now and then cannot be found in the future, this may indicate underlying concerns about the river system itself.

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